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PRINCIPAL INVESTIGATOR: Rafail Kushak, PhD, Dr. Sc.

CONTRACTING ORGANIZATION: Massachusetts General Hospital
Boston, Massachusetts 02114-2621

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Rafail Kushak, PhD. Dr. Sc.

E-mail:rkushak@partners.org

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14. ABSTRACT

The entire microbial population of the duodenal mucosa of subjects with autism was analyzed to determine if there was an overgrowth of a specific populations of bacteria in comparison with unaffected subjects. Patient information, disaccharidase test results, and microbiome analysis were used for group comparison. Duodenal microbiota in autistic individuals was studied for the first time. Study population was represented by 21 autistic subjects and 19 unaffected subjects. Individuals in both groups had GI symptoms. Mucosa-associated microbiota in the duodenum was represented by Bacteroidetes, Firmicutes, and Proteobacteria with no statistically significant difference between groups on fyla level. Numbers of bacteria of Pedobacter genus was significantly higher in subjects with autism but genera Neisseria, Shigella, Blautia, and Enterobacter were less abundant in autistic subjects than in controls. Statistically significant difference between autistic and non-autistic subjects was found for eight bacterial species. There was a correlation between disaccharidase activity (particularly lactase) and duodenal microbiota.

15. SUBJECT TERMS

Autism, duodenal microbiome, DNA extraction, PCR, sequencing, disaccharidases

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Introduction

Children with autistic spectrum disorders (ASD) frequently experience significant gastrointestinal (GI) problems, including abdominal pain, constipation, diarrhea, and bloating. These symptoms exacerbate their behavioral problems. There are speculations that symptoms of malabsorption in patients with autism at least partially are due to a disruption of the indigenous microbiota promoting the overgrowth of potentially pathogenic micro-organisms, such as Clostridia (Bolte, 1998; Finegold et al., 2002, 2011; Song et al., 2004; Parracho et al., 2005). Some of them are recognized toxin-producers, including neurotoxins (Hatheway, 1990**).** However, most of these effects are associated with colon bacteria which normally contain large numbers of predominantly Gram-negative microorganisms. Small intestinal microbiota is represented predominantly by Gram-positive microorganisms, derived from the oropharynx (Simon & Gorbach, 198686; Riordan et al., 2001) and its effect on individuals with autism is understudied. Recent metagenomic analysis by Williams et al. (2011) demonstrated compositional dysbiosis in the ileum of autistic children, decreases in Bacteroidetes, increases in the ratio of Firmicutes to Bacteroidetes, and increases in Betaproteobacteria. Interestingly, expression levels of intestinal disaccharidases and sugar transporters were associated with the abundance of affected bacterial phylotypes. Authors speculate that these results indicate a relationship between human intestinal gene expression and bacterial community structure and may provide insights into the pathophysiology of gastrointestinal disturbances in children with autism.

The overall goal of our study was to analyze the entire microbial population of the mucosa in the upper GI tract of children with autism to determine if there is an overgrowth of specific populations of bacteria in comparison with unaffected children. In aim 1, we analyzed duodenal microbiome of children with and without ASD who have had a biopsy taken for evaluation of GI symptoms. In aim 2, the results of aim 1 we correlated with children's variables including their age, gender, intestinal disaccharidases activity, and GI symptoms. Many children with ASD are given diets and medications that could alter the intestinal microbiota and these pilot data would provide evidence in support of further studies that would look at the relationship between the intestinal microbiota and behavior.

Body

Methods

All autistic and non-autistic individuals had undergone an upper GI endoscopy at the Pediatric GI and Nutrition Unit for evaluation of suspected GI disorders. Duodenal biopsies were taken during the procedure from the second part of the duodenum for routine pathological examination and for disaccharidase activity analysis. Excess tissue was snap frozen, and preserved at -80^{o} C in Digestive Function Laboratory tissue biorepository. It was used for duodenal microbiome analysis.

Intestinal disaccharidase activity analysis included lactase, sucrase, maltase, and palatinase evaluation (Dahlqvist, 1968) and their activity normalization to the level of protein in the biopsy (Bradford, 1976). Microbiome studies included DNA isolation from intestinal mucosa, the 16S rRNA amplification with PCR, and shotgun reads on a 454 Life Sciences Pyrosequencer followed by the computational analysis (Gill et al., 2006; Turnbaug et al., 2007).

Subjects age and enzyme activity are represented as mean±SE. Comparison between groups were performed by two-sided t-test or ANOVA. P values < 0.05 were considered statistically significant.

Microbiome diversity was examined from two perspectives. First, overall richness (i.e., number of distinct organisms present with the microbiome), was expressed as the number of operational taxonomic units (OTUs). Second, overall diversity (which is determined by both richness and evenness, the distribution of abundance among distinct taxa) was expressed as Shannon Diversity. Shannon diversity (*H'*) is calculated using:

$$H' = -\sum_{i=1}^{R} p_i ln(p_i)$$

where R is richness and p_i is the relative abundance of the ith OTU. For both, rarefaction was used to indicate the impact of sampling depth on diversity.

Individual bacterial taxa were screened for group differences using an ANOVA that also included gender and age as factors. Prior to analysis, relative abundances were transformed using a logit transformation. As a somewhat liberal screening process, no adjustment for multiple testing was applied.

Multivariate differences among groups were evaluated using distance based redundancy analysis (dbRDA) (Anderson & Willis, 2003). For the dbRDA, distances among samples first were calculated using UniFrac distances, and then an ANOVA-like simulation was conducted to test for group differences. Weighted UniFrac considers relative abundances whereas unweighted does not.

UniFrac distances were calculated using QIIME (Caporaso et al., 2010), and all other analyses were conducting in R (R Development Core Team. R: A Language and Environment for Statistical Computing, 2011) using the vegan (Oksanen et al., 2011) and labdsv (Roberts, 2010) packages.

Study Subjects

Study groups were represented by 21 subject with ASD and 19 unaffected subjects (controls). Individuals in both groups had GI symptoms including constipation, abdominal pain, and GERD. Constipation was more frequent in autistic group (14 out of 21, 67%) than in control group (3 out of 19, 16%). GERD also prevailed in autistic group
(9 out of 21, 43%) in comparison with controls (9 out of 19, 16%). Abdominal pain was present in three patients with ASD and in four control patients. In autistic group, two patients had esophagitis, one patient had eosinophilic esophagitis, one patient had food allergy, one patient had steatohepatitis, and one patient had proctitis. In control group, three patients had esophagitis, two patients had eosinophilic esophagitis three patients had ulcerative colitis, one patient had Crohn's disease, one patient had autoimmune pancreatitis, and one patient had irritable bowel syndrome. Age of patients in both groups was similar: 14.43±1.07 years in autistic group and 16.05±1.25 years in control group. As was expected, number of males in autistic group (19) was significantly higher (9.5 times) then females (2). In control group number of males and females was close: 10 males and 9 females (Table 1).

Table 1

Study subjects

Subjects	Number	Male	Female	Age, years
Autistics	21	19	2	14.43±1.07
Controls	19	10	9	16.05±1.25

Intestinal disaccharidases

No difference in disaccharidase activity between individuals with and without autism was found. It was expected, since all autistic patients were more than five years old. (Kushak et al., 2011).

Table 2

Enzyme activity

Subjects	Lactase	Sucrase	Maltase	Palatinase
Autistics	15.15±3.84	50.83±6.43	226.71±27.85	15.01±1.65
Controls	10.55±2.06	43.78±6.06	211.77±27.42	14.35±1.52

Duodenal microbiota diversity and composition

Mucosa-associated duodenal microbiome in individuals with ASD was analyzed for the first time and compared with microbiome of un-affected subjects. As we mentioned above, number of organisms was presented in OTUs. Data are presented as a summary statistics and ANOVA analysis. The statistically significant difference between groups of autistic and non-autistic subjects for the number of OTUs was not found (Tables 3 and 4, Figure 1). However, such difference of OTUs was found for the age and gender. Number of OTUs in males was significantly higher than in females.

Table 3. Summary statistics for the number of OTUs, by group.

Variable	**Levels**	**n**	**Min**	**q_1**	**x**	**$\bar{x}$**	**q_3**	**Max**	**s**	**IQR**	**#NA**
OTU	Autistic	14	166.3	178.5	198.7	199.8	214.5	255.9	24.3	35.9	5
	Control	16	88.2	153.5	178.9	167.6	187.6	222.7	35.5	34.1	2
	all	30	88.2	168.0	182.3	182.6	208.7	255.9	34.4	40.8	7

Table 4. Results of the ANOVA for the number of OTUs.

Listed values are p-values for the main effects.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
age	1	8718.50	8718.50	12.15	0.0018
gender	1	4292.71	4292.71	5.98	0.0215
group	1	2590.81	2590.81	3.61	0.0686
Residuals	26	18661.90	717.77		

The diversity index reflect how many types (species) there are in a dataset and simultaneously take into account how evenly they are distributed among those types.

The Shannon diversity index did not show any statistically significant difference between ASD and control groups; however it was significantly different for age and gender. (Tables 5 and 6, Figure 2).

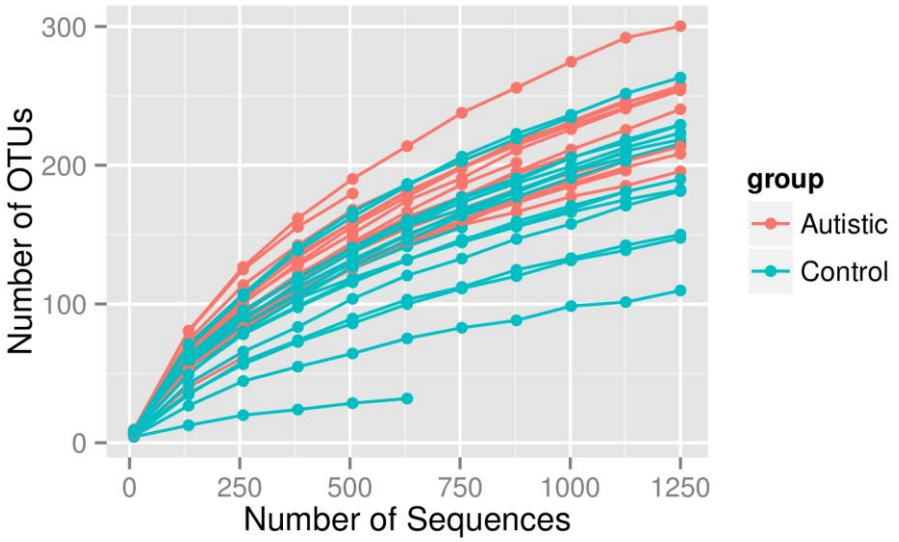
Table 5. Summary statistics for Shannon Diversity, by group.

Variable	Levels	n	Min	q_1	x	$\bar{x}$	q_3	Max	s	IQR	#NA
Shannon. Diversity	Autistic	14	5.1	5.8	6.1	6.1	6.3	7.0	0.5	0.5	5
	Control	16	3.1	5.0	5.5	5.3	5.9	6.5	1.0	0.9	2
	all	30	3.1	5.3	5.8	5.6	6.2	7.0	0.9	0.8	7

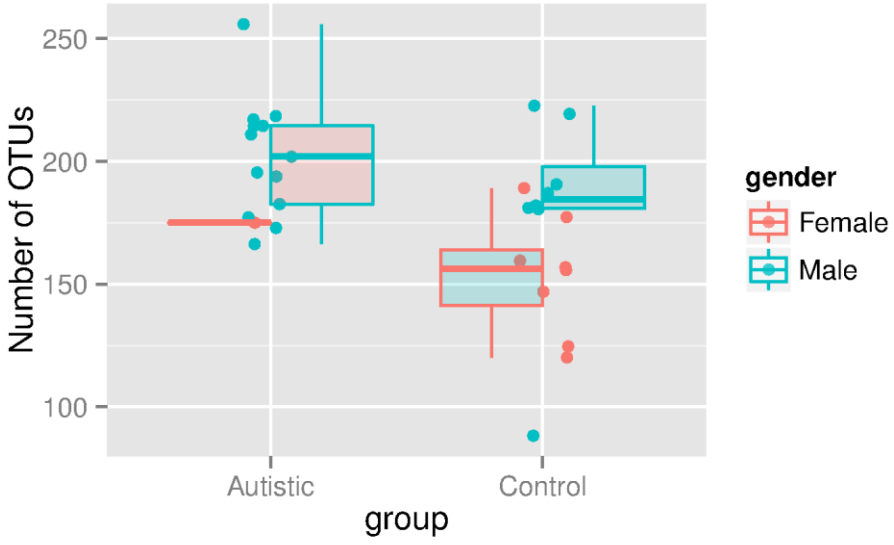
Table 6. Results of the ANOVA for shannon diversity. Listed values are p-values for the main effects.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
age	1	4.06	4.06	7.45	0.0112
gender	1	3.23	3.23	5.93	0.0221
group	1	1.65	1.65	3.03	0.0934
Residuals	26	14.15	0.54		

(a) Rarefaction curves for OTUs



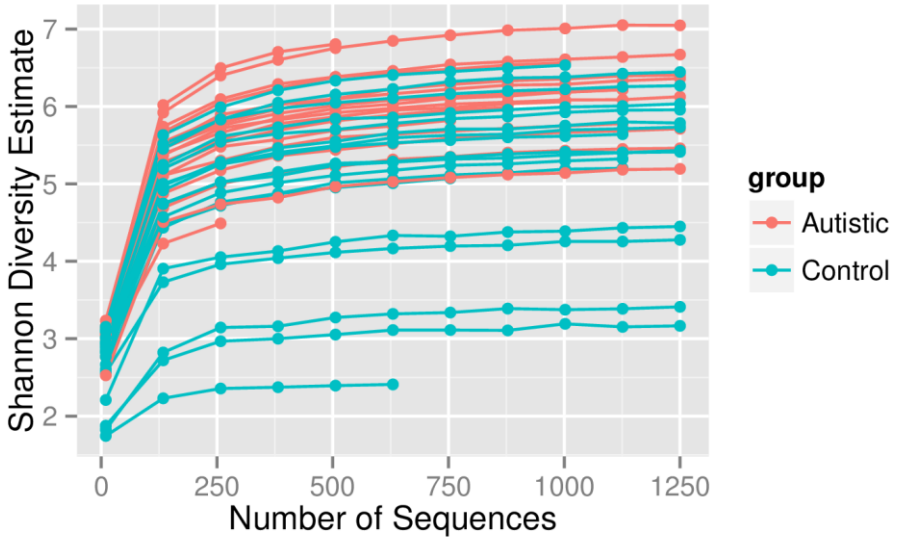
(b)



Boxplot

Figure 1: Number of OTUs within the microbiome (bacteria) data. The number of OTUs is based on 3% divergence, and the number of sequences considered per sample is standardized. Here, individual observations from the same subject are linked via a line.

(a) Rarefaction curves for the Shannondiversity estimate



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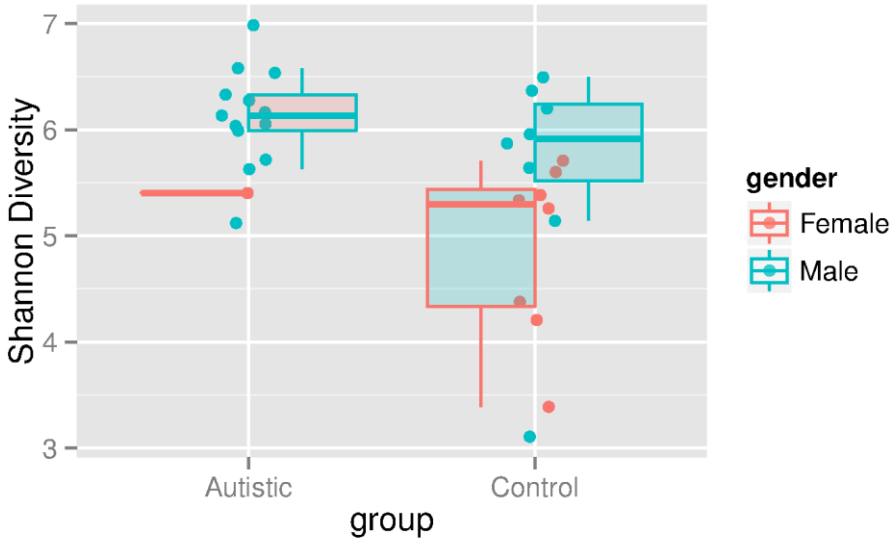


(b) Boxplot

Figure 2: Shannon diversity estimate. The number of sequences considered per sample is standardized, and individual observations from the same subject are linked via a line.

Taxonomic assignments of the sequences showed that mucosa-associated microbiota inthe duodenum consisted of Bacteroidetes, Firmicutes, and Proteobacteria phyla. No statistically significant difference for these phyla was observed between autistic and non-autistic subjects (Table 7).

Table 7. Summary statistics for individual phyla, by group.

Variable	Levels	n	Min	q_1	x	$\bar{x}$	q_3	Max	s	IQR	#NA
Bacteroidetes	Autistic	19	0.00	5.12	10.67	16.27	23.57	73.91	17.25	18.45	0
	Control	18	0.00	11.46	18.35	27.98	44.39	76.21	24.84	32.93	0
	all	37	0.00	5.30	15.53	21.97	29.68	76.21	21.81	24.39	0
Firmicutes	Autistic	19	2.27	24.68	34.21	37.36	52.05	82.28	20.65	27.37	0
	Control	18	0.00	13.35	31.41	31.91	45.34	89.31	24.50	31.99	0
	all	37	0.00	18.52	34.21	34.71	48.39	89.31	22.45	29.87	0
Proteobacteria	Autistic	19	2.66	14.07	21.90	27.73	34.61	93.18	21.28	20.54	0
	Control	18	3.11	13.65	17.75	27.69	43.56	65.12	20.22	29.91	0
	all	37	2.66	13.50	19.67	27.71	40.74	93.18	20.48	27.24	0

Statistically significant difference between groups was found on genus level. (Tables 8 and 9, fig. 3). Bacteria count of Pedobacter genus was significantly higher in subjects with autism but genera Neisseria, Shigella, Blautia, Enterobacter were less abundant in autistic subjects than in controls.

Table 8. Summary statistics for individual genera, by group.

Variable	Levels	n	Min	q_1	x	$\bar{x}$	q_3	Max	s	IQR	#NA
Neisseria	Autistic	19	0	0	0.00	1.55	2.08	7.72	2.70	2.08	0
	Control	18	0	0	2.53	8.09	10.58	50.00	12.99	10.58	0
	all	37	0	0	0.00	4.73	4.55	50.00	9.71	4.55	0
Shigella	Autistic	19	0	0	0.00	0.00	0.00	0.06	0.01	0.00	0
	Control	18	0	0	0.00	0.04	0.00	0.50	0.13	0.00	0
	all	37	0	0	0.00	0.02	0.00	0.50	0.09	0.00	0
Blautia	Autistic	19	0	0	0.00	0.00	0.00	0.06	0.01	0.00	0
	Control	18	0	0	0.00	0.11	0.00	1.52	0.36	0.00	0
	all	37	0	0	0.00	0.05	0.00	1.52	0.25	0.00	0
Enterobacter	Autistic	19	0	0	0.00	0.00	0.00	0.00	0.00	0.00	0
	Control	18	0	0	0.00	0.33	0.07	2.93	0.84	0.07	0
	all	37	0	0	0.00	0.16	0.00	2.93	0.60	0.00	0
Pedobacter	Autistic	19	0	0	0.00	0.16	0.00	2.78	0.64	0.00	0
	Control	18	0	0	0.00	0.00	0.00	0.00	0.00	0.00	0
	all	37	0	0	0.00	0.08	0.00	2.78	0.46	0.00	0

Table 9. Results of the ANOVAs for individual genera. Listed values are p-values for the main effects.

	Age	Gender	Group
Neisseria	0.72	0.36	0.02
Shigella	0.31	0.36	0.05
Blautia	0.37	0.28	0.02
Enterobacter	0.35	0.26	0.00
Pedobacter	0.22	0.09	0.04

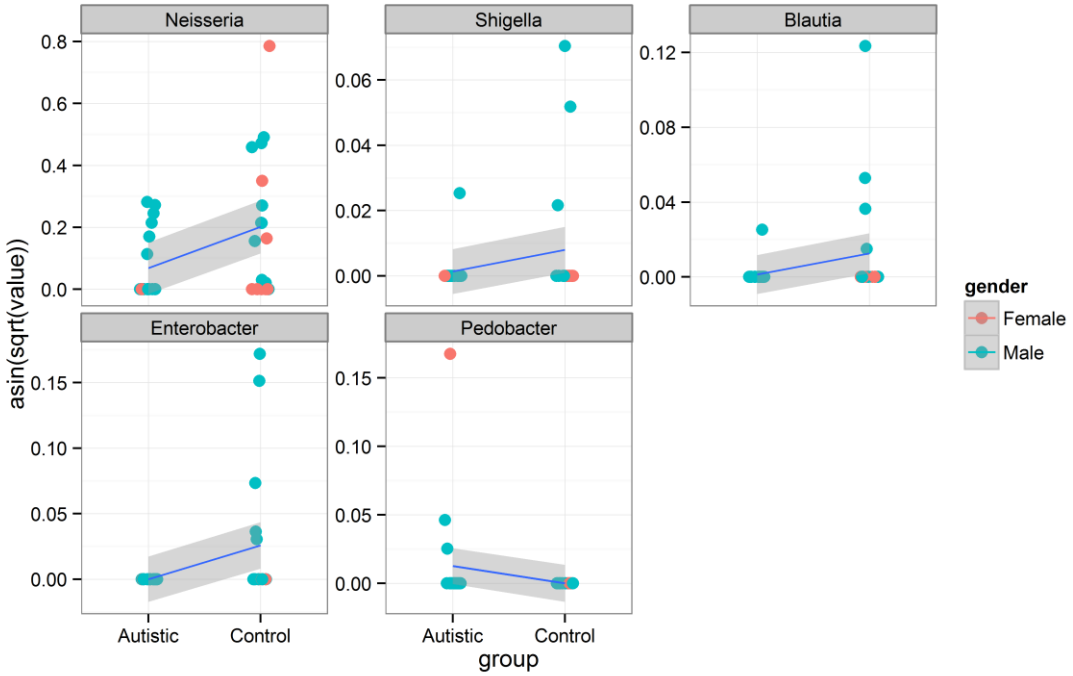


Figure 3. Relative abundance of the genera that had significantly different relative abundances between groups (control vs. autistic). Here, the arcsin transformed observations from each subject are color coded based on gender, and the data are jittered along the x-axis to alleviate overplotting. The blue line connects the the means in each group and indicates the change between groups.

Analysis on species level demonstrated statistically significant difference between autistic and non-autistic subjects for eight species. Autistic subjects demonstrated
decreased number of sequences for Bacteroides. vulgatus, Escherichia.sp, Ruminococcus.gnavus, Neisseria.sp, Blautia.coccoides, and Enterobacter.hormaechei than controls. However, Burkholderia.cepacia and Pedobacter.sp were more abundant in subjects with ASD than in comparison with controls (Tables 10, 11, Fig. 4)

Table 10. Summary statistics for individual species, by group.

Variable	Levels	n	Min	q_1	x	$\bar{x}$	q_3	Max	s	IQR	#NA
Bacteroides.vulgatus	Autistic	19	0	0.00	0.00	3.15	0.02	47.83	11.10	0.02	0
	Control	18	0	0.00	0.00	6.38	6.90	52.72	12.94	6.90	0
	all	37	0	0.00	0.00	4.72	0.64	52.72	11.97	0.64	0
Escherichia.sp	Autistic	19	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0
	Control	18	0	0.00	0.00	0.34	0.00	4.12	1.01	0.00	0
	all	37	0	0.00	0.00	0.17	0.00	4.12	0.72	0.00	0
Burkholderia.cepacia	Autistic	19	0	1.63	4.35	9.71	13.52	54.17	13.22	11.88	0
	Control	18	0	0.00	0.38	7.01	4.33	60.00	16.62	4.33	0
	all	37	0	0.19	2.33	8.40	7.84	60.00	14.82	7.66	0
Ruminococcus.gnavus	Autistic	19	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0
	Control	18	0	0.00	0.00	0.34	0.17	2.60	0.71	0.17	0
	all	37	0	0.00	0.00	0.17	0.00	2.60	0.52	0.00	0
Neisseria.sp	Autistic	19	0	0.00	0.00	1.53	1.97	7.59	2.67	1.97	0
	Control	18	0	0.00	2.40	7.94	10.07	50.00	13.02	10.07	0
	all	37	0	0.00	0.00	4.65	4.55	50.00	9.70	4.55	0
Blautia.coccoides	Autistic	19	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0
	Control	18	0	0.00	0.00	0.02	0.00	0.28	0.07	0.00	0
	all	37	0	0.00	0.00	0.01	0.00	0.28	0.05	0.00	0
Enterobacter.hormaechei	Autistic	19	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0
	Control	18	0	0.00	0.00	0.30	0.03	2.50	0.77	0.03	0
	all	37	0	0.00	0.00	0.15	0.00	2.50	0.55	0.00	0
Pedobacter.sp	Autistic	19	0	0.00	0.00	0.16	0.00	2.78	0.64	0.00	0
	Control	18	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0
	all	37	0	0.00	0.00	0.08	0.00	2.78	0.46	0.00	0

Table 11. Results of the ANOVAs for individual species. Listed values are p-values for the main effects.

	Age	Gender	Group
Bacteroides.vulgatus	0.93	0.44	0.05
Escherichia.sp	0.93	0.23	0.01
Burkholderia.cepacia	0.71	0.81	0.02
Ruminococcus.gnavus	0.21	0.71	0.01
Neisseria.sp	0.70	0.37	0.02
Blautia.coccoides	0.97	0.30	0.03
Enterobacter.hormaechei	0.26	0.26	0.00
Pedobacter.sp	0.22	0.09	0.04

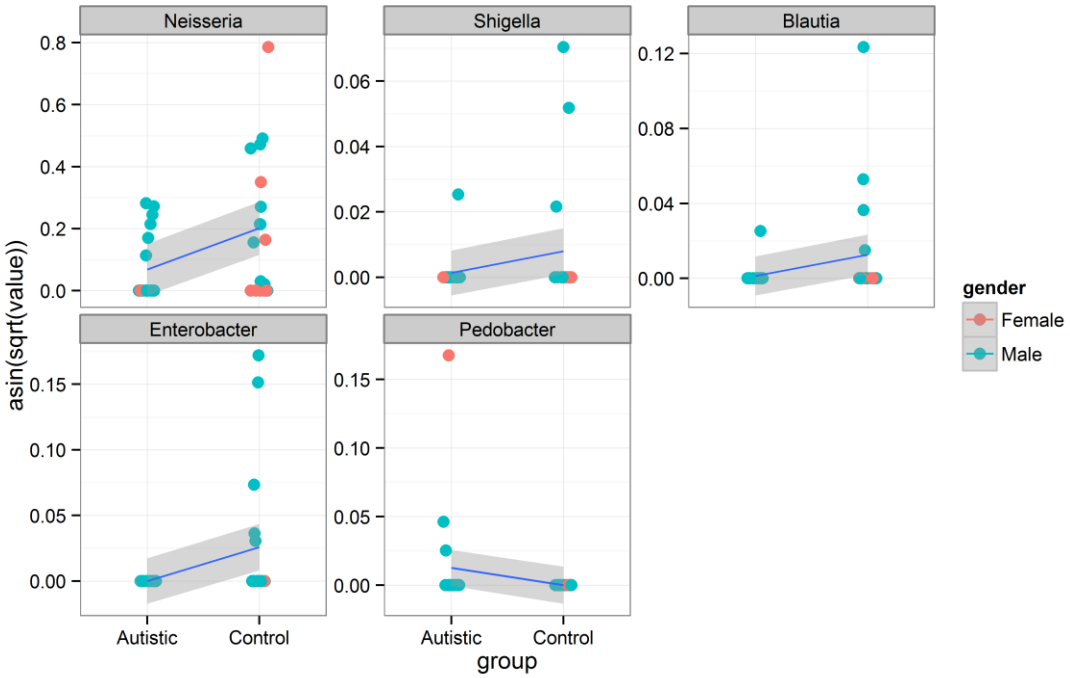


Figure 4. Relative abundance of the genera that had significantly different relative abundances between groups (control vs. autistic). Here, the arcsin transformed observations from each subject are color coded based on gender, and the data are jittered along the x-axis to alleviate overplotting. The blue line connects the the means in each group and indicates the change between groups.

Using the overall predominant genera, clustering analysis was performed to assess the importance of the duodenal gut flora in studied population. The heatmap on Figure. 5 demonstrates the relative abundance of 25 most dominant genera indicating a similar overall composition between autistic and control groups. The left side of the heatmap shows some indication of grouping with higher abundance of Burkholderia, Streptococcus and Prevotella in autistic individuals and Bacteroides in controls. The red color, indicating a high abundance of genus regresses to more orange, yellow, and green tones indicating a decrease in the amount of bacteria (Fig. 5). Based on this information, there appears to be some indication of the gut microflora differing between the autistic and control groups.

On Figure 6, the whole microbiome of autistic subjects and controls is presented in two dimensions. Only taxa with largest contributions are shown. No significant difference between groups can be seen on this biplot.

The heatmaps on Figures 7 and 8 demonstrate relationship between taxa and intestinal disaccharidases activity. On both figures only genus or species that have at least one significant correlation with enzyme activity are represented. Positive correlation is in green and negative correlation is in red. Nine different genera show some correlation with intestinal disaccharidases. On genera level the most significant was correlation with lactase activity (Figure 7).

Analysis of correlation between intestinal enzyme activity and 23 microbiota species demonstrated that only three of them have negative correlation with disaccharidases.
Fourteen species correlate mostly with lactase activity and eight species with sucrase, maltase and palatinase activity (Figure 8).

Problems with sequencing

This study encountered a major problem associated with inability of our original subcontractor at the Institute of Genome Sciences, University of Maryland School of Medicine to sequence the duodenal microbiome from autistic children and controls. These biopsies were taken from children having five completed questionnaires for screening autistic behavior and GI symptoms. We had to find a new subcontractor, the Research and Testing Laboratory, that was chosen based on its performance. Approval to change the sub-contractor was received from DOD on 8/24/12. A new set of 40 biopsies from affected and un-affected children was taken from the tissue biorepository and sent to the Research and Testing Laboratory. However, these biopsies are not supported by the questionnaires on autistic behavior and GI symptoms.

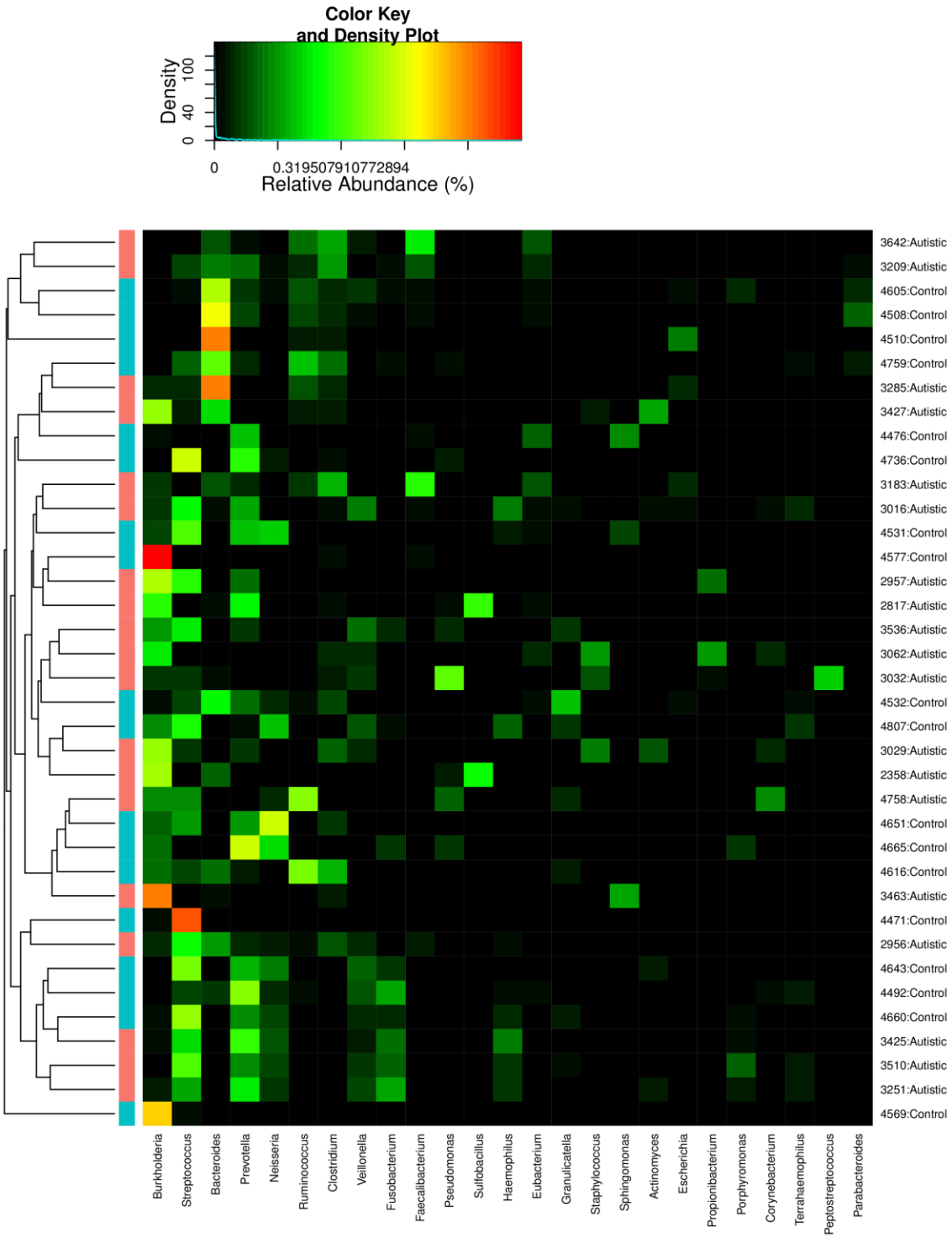
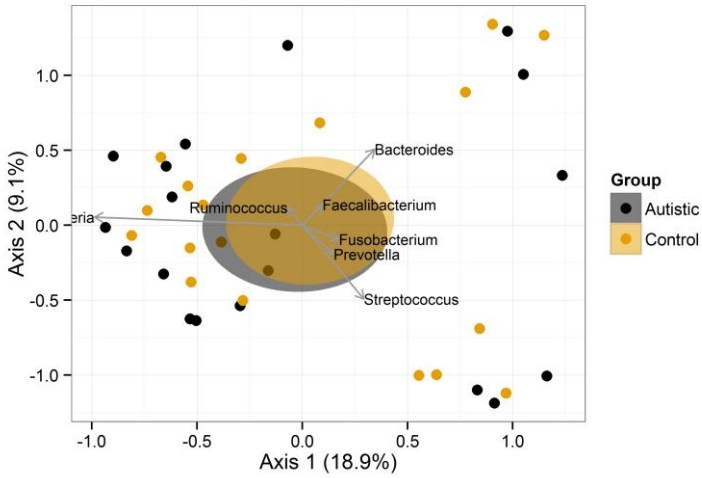
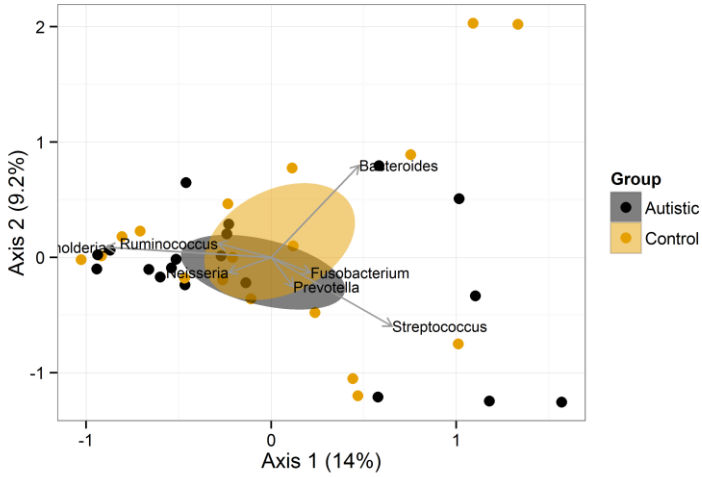


Figure 5: Heatmap summarizing the relative abundance of the 25 most dominant genera in all samples. Samples are sorted based on hierarchical clustering of the Bray-Curtis distances, and group (autistic vs. control) is highlighted via the colors at the tips of the dendrogram. The order of taxa is determined by a hierarchical clustering of Euclidean distances among taxa (dendrogram not shown).



(a) Unweighted Unifrac



(b) Bray- Curtis

Figure 6. Overall Microbiome Biplot of the PCoA, based on presence/absence (unweighted UniFrac) and on relative abundances of OTUs (Bray-Curtis distances). Ellipses represent the 95% confidence interval around group centroid, and differences among groups are not significant. Arrows indicate the contribution of individual taxa to the PCoA axes, and only those taxa with the largest contributions are shown.

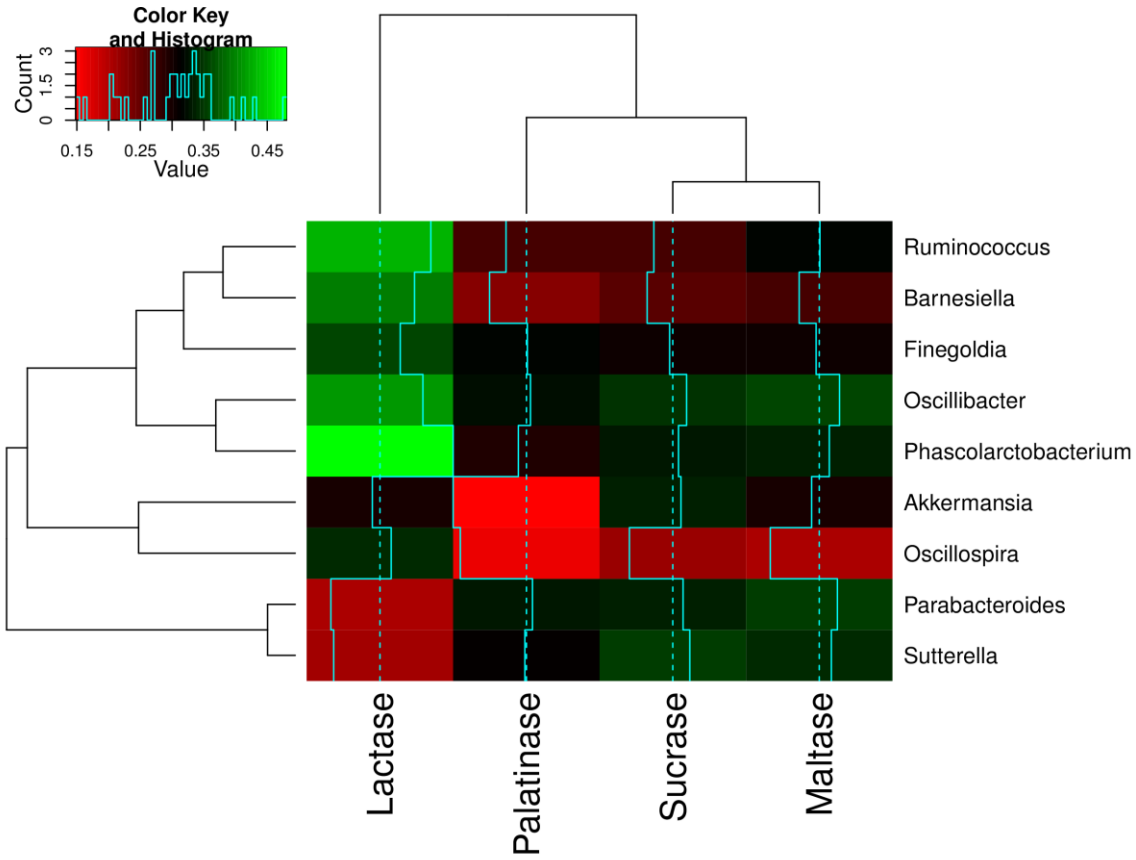


Figure 7. Heatmap summarizing Spearman rank correlations between genera and duodenal disaccharidase activity. Here, only genera that have at least one significant correlation with a disaccharidase are shown. In addition, genera are sorted based on a hierarchical clustering of correlation values.

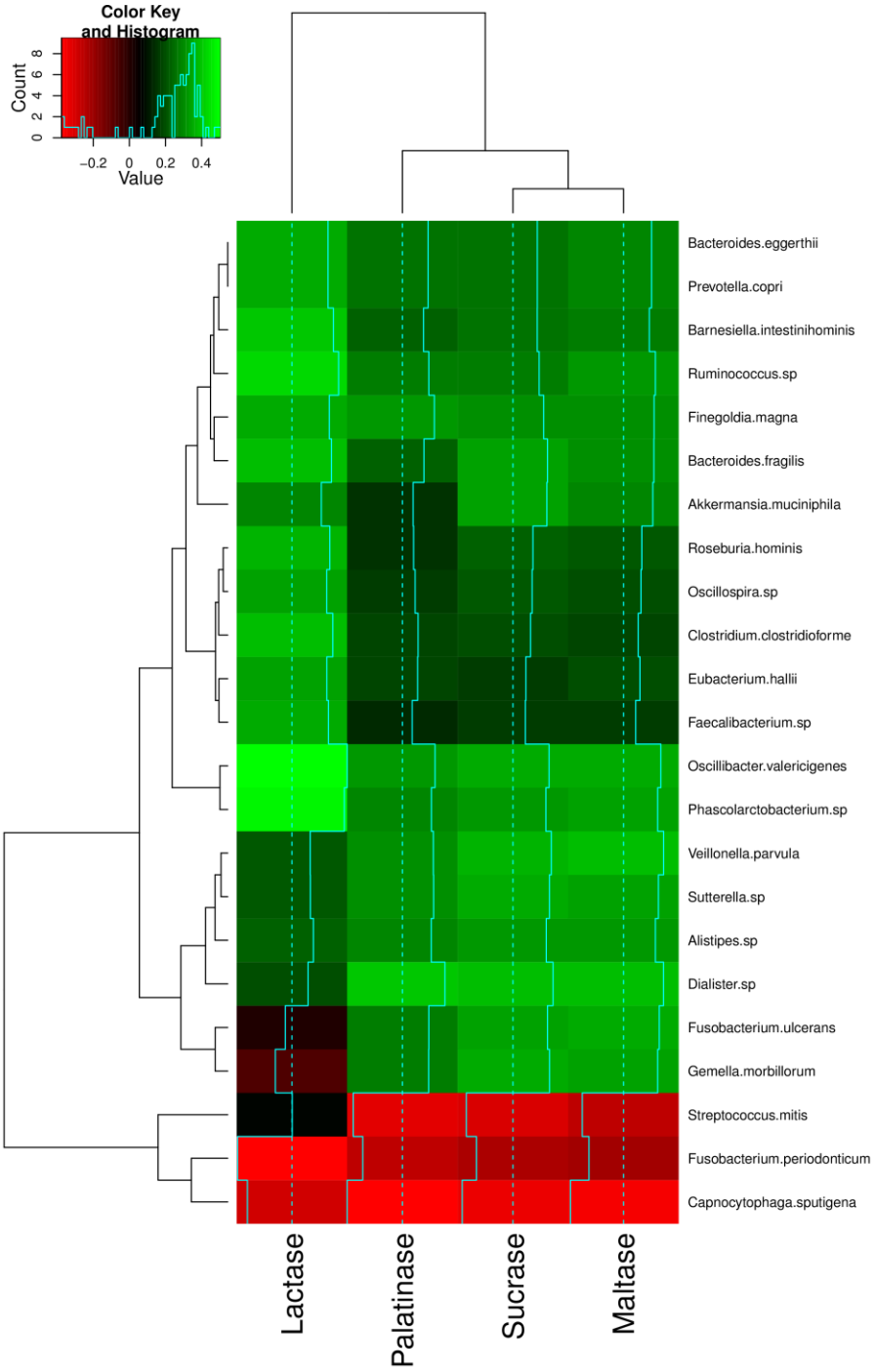


Figure 8. Heatmap summarizing Spearman rank correlations between species and duodenal disaccharidase activity. Here, only species that have at least one significant correlation with a disaccharidase are shown. In addition, species are sorted based on a hierarchical clustering of correlation values.

Key Research Accomplishments

- The entire microbial population of the duodenal mucosa of subjects with autism was analyzed to determine if there is an overgrowth of specific populations of bacteria in comparison with unaffected subjects. Patient information, disaccharidase test results, and microbiome analysis were used for group comparison. Duodenal microbiota in autistic individuals was studied for the first time.

- There were statistically significant differences in duodenal microbiota between autistic and non-autistic subjects on genus and species level, but not on phyla level.

- Correlation between disaccharidase activity (particularly lactase) and duodenal microbiota was found.

Reportable Outcomes

No outcomes to report at the present time

Conclusion

1. There was not statically significant difference between number of OTUs in autistic and non-autistic subjects, however, the number of OTUs in males was significantly higher than in females.

2. Mucosa-associated microbiota in the duodenum is represented by Bacteroidetes, Firmicutes, and Proteobacteria with no statistically significant difference between groups on phyla level.

3. Numbers of bacteria of Pedobacter genus was significantly higher in subjects with autism but genera Neisseria, Shigella, Blautia, Enterobacter were less abundant in autistic subjects than in controls.

4. Statistically significant difference between autistic and non-autistic subjects was found in eight species. Autistic subjects had lower number of Bacteroides. vulgatus, Escherichia.sp, Ruminococcus.gnavus, Neisseria.sp, Blautia.coccoides, and Enterobacter.hormaechei than controls. However, Burkholderia.cepacia and Pedobacter.sp were more abundant in subjects with ASD than in controls.

6. Correlation between disaccharidase activity (particularly lactase) and duodenal microbiota was found.

The significance of these findings is in the process of evaluation and a manuscript is being prepared.

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